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- (71) Applicant (for all designated States except US):
POLYPEPTIDE LABORATORIES A/S [DK/DK];
Herredsvæjen 2, DK-3400 Hillerød (DK).
- (71) Applicants and
(72) Inventors: RASMUSSEN, Jon, H. [DK/DK]; Bud-
dingevej 69, DK-2800 Lyngby (DK). RASMUSSEN,
Palle, H. [DK/DK]; Aldershvilevej 121B, 1th, DK-2880
Bagsvaerd (DK).
- (74) Common Representative: POLYPEPTIDE LABO-
RATORIES A/S; FOMSGAARD, Jens, Herredsvæjen 2,
DK-3400 Hillerød (DK).
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(54) Title: PEPTIDE PURIFICATION

(57) Abstract: A nona- or decapeptide is purified from residual organic solvent by dissolving in a solvent comprising water and at least one C₁-C₃ alcohol followed by precipitation into a vigorously stirred solvent consisting of an alkyl ester of a carboxylic acid, the ester comprising from 3 to 6 carbon atoms, and one or several non-polar compounds selected from hexane, heptane, octane, cyclohexane, methylcyclohexane, and, optionally, of up to 5 % of acetic or propionic acid, isolating the precipitated nona- or decapeptide, followed by washing with a mixture of C₃-C₅ esters and drying, with the proviso that the water content of the solvent comprising water and the at least one alcohol is below 8 % (v/v), and that the volume ratio of the dissolution solvent mixture and the precipitation solvent mixture is 1:10 or higher. Also described is the monoacetate of Ac-D-2Nal-D-4CIPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂.

PEPTIDE PURIFICATION**FIELD OF THE INVENTION**

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The present invention relates to the purification of peptides, in particular intermediate-size peptides, more particularly nona- and decapeptides, such as LHRH-antagonists.

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BACKGROUND OF THE INVENTION

Most intermediate size natural and synthetic peptides are amorphous substances. Many of them have pharmacologically interesting properties, such as many nona- and decapeptides which are LHRH (luteinizing hormone-releasing hormone) antagonists. One particular substance of this kind known only in amorphous form is the synthetic decapeptide of the formula (I)

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Ac-D-2Nal-D-4ClPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂ (I)

which, being a potent LHRH antagonist, has desirable pharmacological properties.

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For use in pharmaceutical preparations it is necessary for the LHRH antagonist (I) and nona- and decapeptides of similar structure to be essentially pure. The raw product obtained in the last step of a multiple-step synthesis is purified by chromatographic and other methods. To eliminate residual solvent from the chromatography a thus purified product usually has to be dissolved in an aqueous medium and freeze-

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dried. This is a costly process producing a voluminous product which is not easy to handle.

A process of purification of an otherwise pure peptide from residual organic solvent by other means than freeze-drying thus is desirable.

OBJECTS OF THE INVENTION

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It is an object of the present invention to provide a process of purification of an otherwise pure peptide of the aforementioned kind from residual organic solvent which avoids freeze-drying.

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It is another object of the present invention to provide such an otherwise pure peptide which is essentially free from residual organic solvent and is not in the form of a cryoprecipitate.

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Further objects of the invention will become obvious from the following summary of the invention, the description of a preferred embodiment thereof, and the appended claims.

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SUMMARY OF THE INVENTION

According to the present invention is provided a process of purification of an otherwise pure peptide, in particular a nona- or decapeptide, most particularly a nona- or decapeptide which is an LHRH antagonist, from residual organic solvent, comprising the following steps:

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- dissolving said otherwise pure peptide in a dissolution solvent mixture comprising water and at least one alcohol selected from methanol, ethanol, propanol, isopropanol;
 - adding the solution of the otherwise pure peptide in said solvent mixture to a vigorously stirred precipitation solvent mixture essentially consisting of one or several polar compounds selected from methyl acetate, ethyl acetate, methyl propionate, ethyl propionate, ethyl isopropionate, butyl acetate, isobutyl acetate, t-butyl acetate, ethyl formate, propyl formate, isopropyl formate, and one or several non-polar compounds selected from hexane, heptane, octane, cyclohexane, methylcyclohexane, and, optionally, of up to 5% of acetic or propionic acid;
 - isolating the precipitated peptide;
 - washing the isolated peptide with one or a mixture of said polar compounds or a solvent or solvent mixture of similar polarity,
 - drying the washed peptide,
- with the proviso that the water content of said solvent mixture comprising water and at least one alcohol is below 8% (v/v), and that the volume ratio of the dissolution solvent mixture and the precipitation solvent mixture is 1:10 or more.

"An otherwise pure peptide" is a peptide which is sufficiently pure for use in a medicine except for volatile impurities which need to be removed or the content of which needs to be substantially reduced. The otherwise pure peptide will normally be a substance having undergone purification by chromatography.

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Preferably the otherwise pure peptide is

Ac-D-2Nal-D-4ClPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂ (I).

According to a first preferred aspect of the invention the water content of the dissolution solvent mixture is below 5% (v/v).

- 5 According to a second preferred aspect of the invention the volume ratio of the dissolution solvent mixture and the precipitation solvent mixture is at least 15, in particular at least 20.
- 10 According to a third preferred aspect of the invention the alcohol of the dissolution solvent mixture is ethanol.

According to a fourth preferred aspect of the invention the polar component of the precipitation solvent mixture is ethyl
15 acetate.

According to a fifth preferred aspect of the invention the non-polar component of the precipitation solvent mixture is heptane.

20 In the following the invention will be described in greater detail by reference to a preferred embodiment thereof which should not be understood to limit the invention.

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DESCRIPTION OF A PREFERRED EMBODIMENT

- Example 1. The fractions containing pure compound (1) (100 g in total) in ethanol-water-acetic acid 40:59:1 (v/v/v)
30 obtained from preparative HPLC by which the synthetically obtained product had been purified were pooled and concentrated in vacuo to an oil which was co-evaporated twice from ethanol. The resulting solid was dissolved in 440 ml of ethanol and the resulting clear solution added over a period

of 20 min to 8.8 L of ethyl acetate/heptane 1:1 (v/v).
Stirring was continued for 1 hour and filtered. The amorphous
product was washed with 3 L of ethyl acetate; it was found
that this removed nearly all heptane. The washed product was
5 dried in a vacuum oven at 40°C at 0.3 bar for 48 hrs.
Elemental analysis of the dried product indicated that the
monoacetate of (I) had been obtained. Cryoprecipitation, in
contrast, produces the corresponding diacetate. In the
following Table analytical parameters of the monoacetate of
10 (I) produced according to the invention are compared with
those of a corresponding lyophilized product (diacetate).

Table. All percentages are by weight

15	lyophilized product	precipitated product	
	Water	3.4 %	3.0%
	Ethanol	<0.024%	<0.024%
	Ethyl acetate	<0.024%	<0.024%
20	Heptane	<0.024%	<0.024%
	Acetic acid (as acetate)	7.5%	3.9%
	HPLC purity	99.8%	99.8%
	Peptide content	88.8%	94.9%
	Loss in filtrate	-	0.3%

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Example 2. Variation of the composition of the precipitation
solvent or solvent mixture; otherwise, procedure as in Example
1. Precipitation in pure ethyl acetate results in from about
3% to about 5% by weight loss of peptide. Precipitation in
30 pure heptane results in a sticky product which is difficult to
filter. A 1:1 (v/v) mixture of ethyl-acetate hexane gives a
product which is easy to filter and dry; in repeated
experiments the loss of peptide was always less than 0.5% by
weight.

Example 3. Variation of water content of the solution of oily product in the dissolution solvent (absolute ethanol); otherwise, procedure as in Example 1. A water content of 10% (v/v) results in a sticky product on precipitation which is difficult to filter. A water content of 15% (v/v) results in an oily product on precipitation. To obtain satisfactory results the water content must not exceed 8% (v/v) but should preferably be kept below 5% (v/v). A water content below 5% is accomplished by co-evaporating the oily product from the chromatography at least twice with ethanol.

Example 4. Variation in precipitation temperature; otherwise, procedure as in Example 1. The precipitation temperature proved to be not critical. It could be varied from 0°C to 20°C without noticeable differences in product yield and morphology.

Example 5. Variation of concentration of (I) in the dissolution solvent; otherwise, procedure as in Example 1. It was found that the concentration of the oily product from the chromatography which had been co-evaporated with ethanol in the dissolution solvent should be as high as possible. Even a concentration of 330 g by weight could be used.

Example 6. Variation of ratio between dissolution solvent and precipitation solvent volumes and other variations; otherwise, procedure as in Example 1. The optimum ratio was found to be about 1:20. It could be shown that ratios from 1:15 to 1:30 gave satisfactory results. A ratio of 1:10 resulted in a sticky product. Precipitation is very fast. The suspension can be filtered 30 min after the last addition of dissolved substance. Washing with ethyl acetate did not result in loss of product but efficiently removed heptane.

CLAIMS

1. A process of purification of an otherwise pure nona- or
5 decapeptide from residual organic solvent, comprising the following steps:
- dissolving the nona- or decapeptide in a dissolution solvent mixture comprising water and at least one alcohol selected from methanol, ethanol, propanol, isopropanol;
 - 10 - adding the solution of the nona- or decapeptide in said solvent mixture to a vigorously stirred precipitation solvent mixture essentially consisting of one or several polar compounds selected from methyl acetate, ethyl acetate, methyl propionate, ethyl propionate, ethyl isopropionate, butyl
15 acetate, isobutyl acetate, t-butyl acetate, ethyl formate, propyl formate, isopropyl formate, and one or several non-polar compounds selected from hexane, heptane, octane, cyclohexane, methylcyclohexane, and, optionally, of up to 5% of acetic or propionic acid;
 - 20 - isolating the precipitated nona- or decapeptide;
- washing the isolated nona- or decapeptide with one or a mixture of said polar compounds or a solvent or solvent mixture of similar polarity,
- drying the washed nona- or decapeptide,
 - 25 with the proviso that the water content of said solvent mixture comprising water and at least one alcohol is below 8% (v/v), and that the volume ratio of the dissolution solvent mixture and the precipitation solvent mixture is 1:10 or more.
- 30 2. The process of claim 1, wherein the water content of said solvent mixture comprising water and at least one alcohol is below 5% (v/v).

3. The process of claim 1, wherein said nona- or decapeptide is an LHRH antagonist.
4. The process of claim 3, wherein said nona- or decapeptide is Ac-D-2Nal-D-4ClPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂ (I).
5. The process of claim 4, wherein Ac-D-2Nal-D-4ClPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂ (I) is obtained in form of the monoacetate.
6. The process of claim 1, wherein the water content of the dissolution solvent mixture is below 5% (v/v).
7. The process of claim 1, wherein the volume ratio of the dissolution solvent mixture and the precipitation solvent mixture is at least 15.
8. The process of claim 1, wherein the alcohol of the dissolution solvent mixture is ethanol.
9. The process of claim 1, wherein the polar component of the precipitation solvent mixture is ethyl acetate.
10. The process of claim 1, wherein the non-polar component of the precipitation solvent mixture is heptane.
11. The monoacetate of Ac-D-2Nal-D-4ClPhe-D-3Pal-Ser-MeTyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂.

INTERNATIONAL SEARCH REPORT

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PCT/SE 02/05581

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 1/30, C01K 7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPO-INTERNAL, PAJ, CHEM.ABS.DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0018423 A1 (LABORATOIRE THERAMEX), 6 April 2000 (06.04.00), page 5 - page 6, claims 9 and 30 --	11
A	WO 0055190 A1 (ASTA MEDICA AG), 21 Sept 2000 (21.09.00), claim 13 --	1-11
A	EP 0136728 A2 (SHELL INTERNATIONALE RESEARCH MAATSCHAPPIJ B.V.), 10 April 1985 (10.04.85) --	1-10
A	EP 0955308 A1 (DEGUSSA-HÜLS AKTIENGESellschaft), 10 November 1999 (10.11.99) --	1-10

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

IDA CHRISTENSEN/BS
Telephone No. +46 8 782 25 00

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 9110677 A1 (BASF AKTIENGESELLSCHAFT), 25 July 1991 (25.07.91)</p> <p style="text-align: center;">-- -----</p>	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0018423 A1	06/04/00	AT 218358 T AU 6199999 A BR 9914125 A CA 2344470 A CN 1324241 T CZ 20011173 A DE 69901721 D,T DK 1117420 T EE 200100196 A EP 0998940 A EP 1117420 A,B SE 1117420 T3 ES 2178478 T HU 0103649 A IL 142112 D JP 2002525336 T NO 20011576 A PL 346939 A PT 1117420 T SI 1117420 T TR 200100887 T ZA 200102452 A	15/06/02 17/04/00 19/06/01 06/04/00 28/11/01 12/09/01 09/01/03 16/09/02 17/06/02 10/05/00 25/07/01 16/12/02 28/02/02 00/00/00 13/08/02 28/03/01 11/03/02 31/10/02 00/00/00 00/00/00 26/03/02
WO 0055190 A1	21/09/00	AU 3288700 A BG 106008 A BR 0009472 A CN 1348462 T CZ 20013318 A DE 19911771 A EP 1163264 A HU 0200363 A NO 20014486 A SK 13232001 A TR 200103339 T ZA 200107753 A	04/10/00 28/06/02 27/11/01 08/05/02 15/05/02 28/09/00 19/12/01 29/06/02 02/11/01 09/05/02 00/00/00 13/05/02
EP 0136728 A2	10/04/85	SE 0136728 T3 AT 26986 T AU 569896 B AU 2997684 A CA 1228041 A DE 3463509 D DK 165411 B,C DK 316984 A GB 8317697 D JP 1874334 C JP 5085559 B JP 60036498 A NZ 208701 A US 4532212 A	15/05/87 25/02/88 03/01/85 13/10/87 00/00/00 23/11/92 30/12/84 00/00/00 26/09/94 07/12/93 25/02/85 30/10/87 30/07/85
EP 0955308 A1	10/11/99	DE 19813849 A JP 11310595 A US 6258933 B	30/09/99 09/11/99 10/07/01

INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9110677 A1	25/07/91	AT 111481 T	15/09/94
		CA 2067790 A	19/07/91
		DE 4001238 A	25/07/91
		DE 59007173 D	00/00/00
		DK 511221 T	17/10/94
		EP 0511221 A,B	04/11/92
		SE 0511221 T3	
		JP 5503511 T	10/06/93
		US 5304310 A	19/04/94